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STUDY OF ANTIOXIDANT ENZYMES AND MITOCHONDRIAL DNA t-RNA^{LEU} (UUR) MUTATION IN MITOCHONDRIAL ENCEPHALOMYOPATHY LACTIC ACIDOSIS AND STROKE LIKE EPISODES (MELAS)

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Absract: The present study was attempted to estimate the level of antioxidant enzymes, free radicals and Mitochondrial DNA 3243 A/G mutation in patients of MELAS syndrome. All standard methods were used to evaluate oxidative stress and mitochondrial DNA mutation. There was alteration in the level of antioxidant enzymes and no mutation was observed. Although there was alteration in the activities of antioxidant enzymes, no mutation was detected in the mitochondrial DNA.
Keywords: Oxidative stress, Antioxidant enzymes, Free radicals, Mitochondrial DNA.

Introduction: Mitochondria are dynamic organelles that are found almost in every cell and produces more than 90% of our cellular energy by oxidative phosphorylation[1]. Energy production is the result of two metabolic processes- the tricarboxylic acid cycle (TCA) and the electron transport chain (ETC). The TCA cycle converts carbohydrates and fats into some ATP and also produces the co-enzymes NADH and FADH that carry electrons to the ETC. During the transfer of the electrons from the respiratory complexes, single electrons sometimes escape and result in reduction of molecular oxygen to form free radicals like ONOO-, OH• ion, O• superoxide anion, H_2O_2 etc; also called as reactive oxygen species (ROS) which are highly reactive and attack the biological molecules like DNA (Deoxyribonucleic Acid), lipids and proteins and alter their structure and function by causing damage to them[2,3].

Mitochondrial DNA is in close proximity to the site of ROS generation and is not protected by histones, so it is vulnerable to mutations. Mutations in such mitochondrial genes often lead to complex, multisystemic disorders referred to as mitochondrial diseases[4]. Mitochondrial dysfunction is often associated with neurodegenerative diseases, cardiomyopathy, diabetes, cancer etc. Oxidative stress is associated with various diseases, one such disease caused by mutation in the mitochondrial DNA is Mitochondrial Encephalomyopathy Lactic Acidosis and Stroke-like episodes (MELAS). Elevated ROS generation is also involved in the pathogenesis of MELAS (Mitochondrial Encephalomyopathy Lactic Acidosis and Stroke-like episodes), and other mitochondrial diseases[5].

2. Materials and Methods:

Sample collection:

This study included 12 samples; six samples of healthy subjects as control and six samples of patients. The samples were collected after taking a consent from the patients. All the samples of the patients were taken from "Swami Vivekanand Medical Mission Hospital" Parsodi, Nagpur. Samples of patients were collected based on their symptoms of MELAS.

Sample preparation:

2 ml of blood samples were collected in EDTA vacutainer tubes; from this 0.3 ml of blood sample was used to isolate DNA and the remaining blood samples were centrifuged at 3000×g for 15 minutes for the collection of plasma. The plasma samples were recentrifuged at 3000×g for same time and plasma were collected in new vials to avoid the remains of blood cells. All the samples were stored at -20°C for further analysis.

Enzymatic analysis:

The concentration of various antioxidant enzymes was estimated by using standard methods. Catalase was measured according to the method given by Aebi et al. (1983)[6]. For SOD , the addition of 2mM cyanide in the plasma inhibited Cu/Zn SOD but Mn/ FeSOD remained unaffected, while the addition of 50 micro L ice cold Choroform/Ethanol inactivated Mn/Fe SOD[7,8]. Both Cu/Zn SOD and Mn/Fe SOD were estimated as per the method of Marklund and Marklund (1974)[9]. Nitric Oxide was estimated according to Green's et al (1982)[10]. TBARS (Thio Barbituric Acid Reactive Substances) was estimated by using the method given by J. Stocks et. al. (1971)[11,12]. The concentration of protein was measured by

using the method given by Lawery O.H. et. al. (1951)[13].

Isolation of Mitochondrial DNA and Identification of Mutation:

DNA was isolated by using whole blood DNA isolation Kit (Ge Nei catalogue No: 612102300011730). Total 581 nucleotides containing DNA was amplified by using the ready to use Master Mix Bioline (Catalogue No.BIO-33057) by PCR (Polymerase Chain Reaction) method. Mutation analysis was done as per the method given by Ouweland J.M.W. [14]. Primers synthesis was done through IDT scientific technologies. PCR reactions were then carried out in the 12 µL reaction mixture comprised of 1 µL of DNA template (100ng), 2µL Forward primer (100ng), 2µL reverse primer (100ng) and 7µL of the Bioline master mix. Protocol consisted of, incubation at 94˚C for 3 minutes, followed by 94˚C for 30 seconds, 55˚C for 30 seconds, 72˚C for 45 seconds and a final incubation at 72˚C for 5 minutes in Biorad Thermal Cycler (USA). Amplified samples were then proceeded for performing RFLP (Restriction Fragment Length Polymorphism);digested by the Restriction Enzyme ApaI GeNie at 37˚C for approximately 24 hours. The subsequent amplicons were then electrophoresed by using 2% agarose gel and visualized by ethidium bromide, with DNA ladder(100 to 1000 bp) GeNei (Catalogue No. GeNei 612652671001730) in the Gel-Doc system (Bio-Rad USA). If the digested amplicons give two bands each of 367 bp and 214 bp containing DNA fragments indicates that A/G mutation is present at position 3243 in mitochondrial DNA. If only a single band of 581 bp DNA appears it indicates an absence of the mutation.

Statistical analysis:

All the statistical analysis was done in MS Excel (2007). All the results were expressed in Mean ± SD. The two tailed probability student's T test was used to differentiate between the healthy subjects and the patients, assuming unequal variance. P< 0.05 was a standard for significance difference.

3. Result:

This study shows that, as compared to the control subjects the activity of catalase is found to be increased in MELAS patients ($0.029 < 0.05$)[Table 1, Fig (A)] likewise an increase in the activity of Mn/FeSOD has been reported in MELAS patients ($0.025 < 0.05$)[Table 1, Fig (B)] also the activity of Cu/ZnSOD is more in MELAS patients ($0.02 < 0.05$) [Table 1, Fig (C)]. The activity of lipid peroxidation is equal in both normal control individuals as well as MELAS patients ($0.3 > 0.05$)[Table 1, Fig (D)]. The concentration of nitric oxide was more in MELAS patients ($0.004 < 0.05$)[Table 1, Fig(E)].

Table 1 : A Statistical analysis of various antioxidant enzymes, lipid peroxidation and nitric oxide.

PARAMETERS	GROUPS	MEAN ± SD	P-VALUE
Catalase	Control	47.78 ± 17.98	
(Units/Mg protein/ML)	MELAS	82.52 ± 27.65	0.029
Mn/Fe SOD	Control	19.53 ± 3.78	
(Units/Mg protein/ML)	MELAS	29.545 ± 7.82	0.025
Cu/Zn SOD	Control	20.03 ± 3.312	
(Units/Mg protein/ML)	MELAS	28.9 ± 7.16	0.02
MDA	Control	0.00004 ± 0.00006	
(nM × 10^{-3}/ML)	MELAS	0.00005 ± 0.00007	0.3
NO	Control	65.64 ± 113.29	
(nM × 10^{-2}/ML)	MELAS	150.39 ± 79.08	0.004

Figure 2 denotes 3243A/G mutation analysis in both control subjects and MELAS patients. Total 581 base pair nucleotide was amplified and digested by Apa1 restriction enzyme. After running in 2% agarose gel if the amplicons showed two bands each of 367 bp and 214 bp, will indicate the presence of 3243A/G mutation in the mitochondrial DNA. A single band of 581bp will represent an absence of the mutation, the present study showed an absence of the 3243 A/G mutation in the mitochondrial DNA.

4. Discussion:

The levels of antioxidant enzymes in both healthy control subjects and MELAS patients were estimated. According to the result the activity of catalase was found to be increased in MELAS patients so no oxidative stress was developed. Catalase is an important antioxidant enzyme which is found in all cells and play an important role in converting hydrogen peroxide into water molecule and oxygen and also plays a vital role in scavenging free radicals[15].

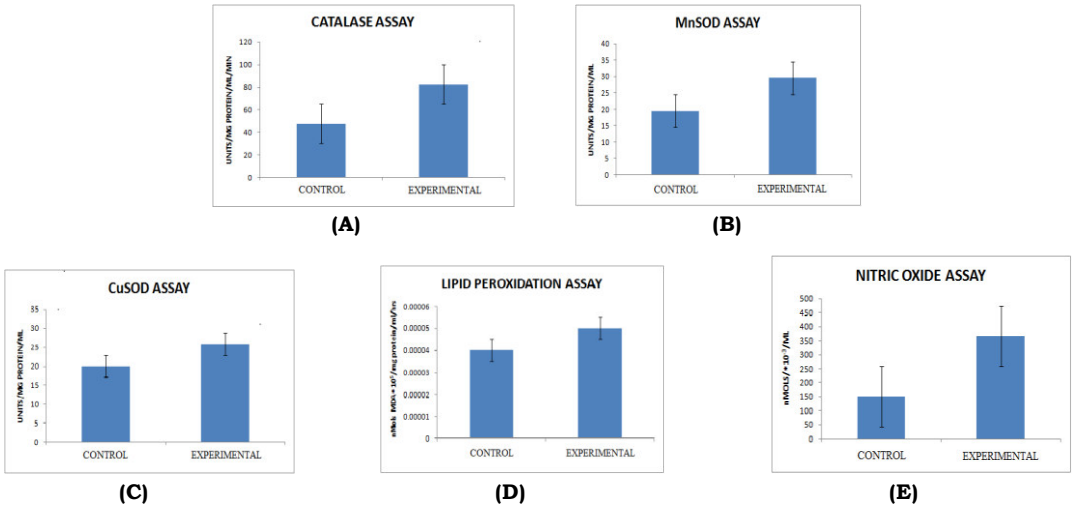

Figure 1: The activity of various antioxidant enzymes,that includes catalase which is represented by a graph (A), the Mn/Fe SOD and Cu/Zn SOD are represented by (B) and (C) ; the concentration TBARS shown by (D) likewise the concentration of Nitric Oxide shown by (E) in normal control subjects and in patients of MELAS. Normal healthy individuals represented by control and MELAS patients are represented by Experimental.

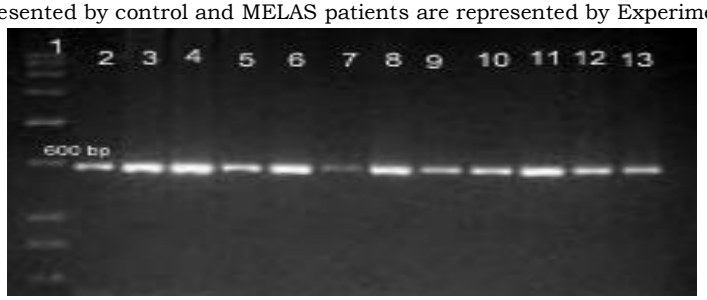

Figure 2: This panel shows the identification of the A/G mutation at position 3243 in mitochondrial genome. An intact 581bp band containing DNA was observed where well 1 represents marker ranging from 100bp to 3000bp. Well number 2 to 7 represents DNA amplification of controls and from well number 8 to 13 denotes mutation identification in patients.

Another antioxidant enzyme, Manganese/ Iron Superoxide Dismutase and Copper/ Zinc containing Superoxide Dismutase also play an important role in scavenging the free radicals that catalyses the dismutation of the highly reactive superoxide anion to hydrogen peroxide molecule. The main aim of the Superoxide Dismutase enzyme is to eliminate the superoxide anion that cause damage to cells, DNA and other biological organelles[16]. This study shows a significant increase in the activity of Mn/Fe SOD and Cu/Zn SOD in MELAS patients.

Lipid peroxidation causes increase in the membrane permeability which leads to cell damage. It is used to establish the oxidative stress by determining the balance between free radicals and antioxidant enzymes [17]. This study did not show altered concentration of MDA in MELAS patients and control individuals. Nitric oxide may react with superoxide anion to form peroxinitrite molecules, that can damage the cellular mechanism. So it is important to maintain the concentrations of free radicals like nitric oxide and superoxide radicals[18]. In the present study a significant increase in the concentration of nitric oxide was observed in MELAS patients.

Mutations in mitochondrial DNA are more common because mtDNA mutates 10-17 times faster than the nDNA as the chromatin and histones are absent and also due to the continuous generation of ROS and the lack of DNA repair mechanisms[19]. Mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS) syndrome is oftently occurring Mitochondrial disease. Pathogenic mutations in most of the 22tRNA genes have been specified among them the most common tRNA mutation is the tRNA Leu A3243G, which is associated with MELAS syndrome[20]. Other tRNA Leu mutations like the G3244A, T3258C, C3256T, T3271C, T3291C and the mutations in the tRNA His and tRNA Val are also linked to MELAS syndrome. In the present study no 3243 A/G mutation was observed in the suspected patients(fig.2)

5. Conclusion:

The moto of this study, was not to find out the maternal inheritance of the 3243A/G

mutation but only the identification of 3243A/G mutation in patients who were showing the symptoms of MELAS. This study did not report the 3243A/G mutation. But as this study corroborating the altered antioxidant enzymes status and altered concentration of nitric oxide, it can hypothesised that other mitochondrial DNA mutation may be possible.

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